

# Effects of myo-inositol concentration on growth and pigments of *Nannochloropsis oculata* culture

## Myo-inositol konsantrasyonunun *Nannochloropsis oculata* kültüründe büyüme ve pigmentleri üzerine etkileri

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**Abstract:** Inositols are used as growth promoting agents over plants. But microalgae are different from higher plant especially photosynthetic efficiency and productivity. According to the results of this study, myo-inositol addition to the culture medium of *Nannochloropsis oculata* provides higher cell densities. 100 mg L<sup>-1</sup> myo-inositol added experimental group was reached to 1.42-fold cell mL<sup>-1</sup>, while the 500 mg L<sup>-1</sup> myo-inositol added group was reached to 1.28-fold cell mL<sup>-1</sup> than the control group. Mean chlorophyll  $\alpha$  per cell amounts were calculated for experimental groups and control groups as 0.052 pg cell<sup>-1</sup> and 0.053 pg cell<sup>-1</sup>, respectively. Mean total carotene per cell amounts were calculated for all groups as 0.016 pg cell<sup>-1</sup>. These results show that no difference was occurred between all groups by chlorophyll  $\alpha$  and total carotene amounts per cell. This study shows that myo-inositol use in microalgae production may provide higher yields.

**Keywords:** carotene, chlorophyll, microalgae, myo-inositol, *Nannochloropsis oculata*, pigments

**Öz:** İnositoller, bitkilerde büyüme artırıcı olarak kullanılmaktadır. Ancak, mikroalgler yüksek bitkilerden özellikle fotosentetik etkinlik ve üretkenlik açısından farklıdır. Bu çalışmada elde edilen verilere göre *Nannochloropsis oculata* kültür ortamına myo-inositol ilave edilmesi daha yüksek hücre yoğunluğuna neden olmaktadır. 100 mg L<sup>-1</sup> myo-inositol ilave edilen grup kontrol grubuna göre 1,42 kat hücre mL<sup>-1</sup>, 500 mg L<sup>-1</sup> myo-inositol ilave edilen grup ise kontrol grubuna göre 1,28 kat hücre mL<sup>-1</sup> yoğunluğa ulaşmıştır. Hücre başına ortalama klorofil  $\alpha$  değerleri ise deneme grupları ve kontrol grubu için sırasıyla 0,052 pg hücre<sup>-1</sup> ve 0,053 pg hücre<sup>-1</sup> olarak hesaplanmıştır. Hücre başına ortalama toplam karoten miktarları ise tüm gruplar için 0,016 pg hücre<sup>-1</sup> olarak hesaplanmıştır. Bu sonuçlar gruplar arasında hücre başına klorofil  $\alpha$  ve toplam karoten miktarlarının değişmediğini göstermektedir. Bu çalışma, mikroalg üretiminde myo-inositol kullanımının daha fazla ürün elde edilmesini sağlayabileceğini ortaya koymaktadır.

**Anahtar kelimeler:** karoten, klorofil, mikroalg, myo-inositol, *Nannochloropsis oculata*, pigmentler

## INTRODUCTION

Microalgae are indispensable sources for biotechnological products of different industrial applications such as feed, health foods, natural colorants, pharmaceuticals and bioenergy (Chu, 2012; Dixit and Suseela, 2013; Mortensen, 2006; Pulz and Gross, 2004). Also, essential feed source for all growth stages of bivalves and for larvae of some crustaceans and fish species in aquaculture as used directly in larval tanks. In this aquaculture feed chain, important nutrients from microalgae are transferred to higher trophic levels via intermediary zooplankton (Brown et al., 1999; Vismara et al., 2003).

The microalga *Nannochloropsis oculata* is important in aquaculture due to its nutritional value. It belongs to the class Eustigmatophyceae, which species that contain a high amount of fatty acids and pigmentation (Brown et al., 1999; Otero et al.,

1997). The nutritional value of microalgae is related to their biochemical composition, especially the lipid and fatty acid compositions. Biochemical composition is alterable significantly through culture conditions, especially depending on the culture medium, temperature and light conditions (Durmaz et al., 2008; Richmond, 2004). The pigment composition of algae from the genus *Nannochloropsis* is characterized by chlorophyll  $\alpha$ , beta-carotene, violaxanthin and vaucherixanthin as major pigments. Pigments are involved in light harvesting reaction and their amounts are differable that are associated with growth conditions, especially medium, high light intensity and high salinity (Lubian et al. 2000, Sanchez et al., 2005). Inositol is a chemical compound with formula C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>, a six-fold alcohols of cyclohexane. It exists several stereoisomers and the most prominent form is myo-inositol.

Myo-inositol and its multifunctional position in plant biochemistry and physiology have already been described (Loewus and Loewus, 1983; Morre et al., 1990). Biosynthesis and function of several phosphoinositide stereoisomers have investigated in terms of signaling and plant growth which supports the essential importance of inositol lipids in plants (Stevenson et al., 2000).

The main problems of microalgae production are low productivity, low quality, and high production cost. Main purposes of microalgae production in the aquaculture are that increment of growth rate and valuable metabolites such as pigment and fatty acids.

Present study was undertaken to examine the effect of myo-inositol on growth and pigments of *N. oculata*. Studies of the effects of different concentration of myo-inositol on pigments may give important information on species metabolism. Moreover, this knowledge is also important for optimizing the yield of marine aquaculture production. Using myo-inositol may provide to obtain more biomass and more pigments.

## MATERIAL AND METHODS

### Microalgae

*N. oculata* (CCAP 849/1) was obtained from The Culture Collection of Algae and Protozoa (CCAP), Scotland.

### Culture conditions

6 L flat-bottom flasks were used in *N. oculata* experiments. Cultures were kept illuminated under fluorescent lamps (Philips Master TL-D Reflex 36W/865 1SL/25) at 116  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$  irradiance level of the surface of flask with 24:0 h (L:D) photoperiod. The cultures were stirred by air without  $\text{CO}_2$  addition. 0.2  $\mu\text{m}$  Sartorius Midisart 2000 filters were used to avoid contamination by aeration. The temperature was maintained at  $20 \pm 1^\circ\text{C}$  by the air-conditioner. 2-fold concentration of F/2 medium was used (Guillard, 1975). In the experiment, sterilized seawater was used with 35  $\text{g L}^{-1}$  of salinity. All solutions except vitamin solution were autoclaved at  $121^\circ\text{C}$  for 20 min. The vitamin solution was sterilized with 0.2  $\mu\text{m}$  filter.

### Experimental Design

Myo-inositol were purchased from Sigma-Aldrich (USA). These inositol derivatives were dissolved in F/2 medium and filtered through a 0.2  $\mu\text{m}$  membrane filter (Whatman Anodisc 47 mm).

100  $\text{mg L}^{-1}$  and 500  $\text{mg L}^{-1}$  myo-inositol concentrations with the control group were chosen in this study and experiments were done in triplicate.

## Analysis

The samples of microalgae were harvested daily for cell count and analysis. Cell number was measured via Improved Neubauer hemocytom eter and at the same time, contamination was checked daily through visual observation. Specific growth rates ( $\mu$ ) were calculated by this equation (Durmaz and Erbil, 2017);

$$\mu = \frac{\ln(N_t) - \ln(N_0)}{t - t_0}$$

Where  $N_t$  is biomass as cell number at the time ( $t$ ) and  $N_0$  is the beginning biomass cell number at a time  $t_0$ .

Chlorophyll  $a$  and total carotene pigments of *N. oculata* were measured by spectrophotometry. 5 mL of samples from each culture were taken and were centrifuged at 3500 rpm for 10 minutes, then supernatants were removed. After that, 5 mL of methanol and glass powder were added to the tubes then were mixed by using vortex. After homogenization with mechanical homogenizer, samples were kept in ultrasonic bath for 10 minutes at  $65^\circ\text{C}$ . Lastly, samples were mixed over again and were centrifuged at 3500 rpm for 10 minutes. Supernatants were taken for spectrophotometric analysis. Calculations were completed according to;

Chlorophyll  $a$  ( $\mu\text{g mL}^{-1}$ ) = 13.9  $A_{665}$  (Sanchez et al., 2005)

$A_{665}$  wavelength 665 nm absorbance value

Total carotene ( $\mu\text{g mL}^{-1}$ ) = 4.5  $A_{478}$  (Zou and Richmond, 2000)

$A_{478}$  wavelength 478 nm absorbance value

All of the reagents were obtained from Merck (Darmstadt, Germany) unless otherwise stated and the standards were from Sigma-Aldrich (St. Louis, USA).

The Kolmogorov-Smirnov test was used to verify the normality and homogeneity of variances. Data were analyzed using ANOVA. The sources of significant differences were determined using the Tukey test (Zar, 1999).

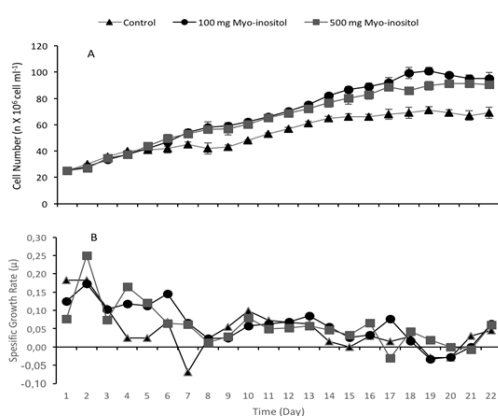
## RESULTS

The pH of the nutrient medium was not changed after adding Myo-inositol and the pH ranges were between 7.5 and 8.5 along to culture time. The air was given in the middle of the flasks, so that the homogenous distribution of the cells and nutrient medium were fully ensured. It is observed that cells sizes were not changed (approximately 2  $\mu\text{m}$  diameter).

### Cell Densities

In this study, initial density of *N. oculata* trials were arranged as  $25 \times 10^6$  cell  $\text{mL}^{-1}$ . After inoculation, the lag phase was not observed. The effects of myo-inositol concentration on the cell number of *N. oculata* are shown in Figure 1A. 100  $\text{mg L}^{-1}$  and 500  $\text{mg L}^{-1}$  concentrations of myo-inositol were used in the medium, the maximum cell densities were reached to

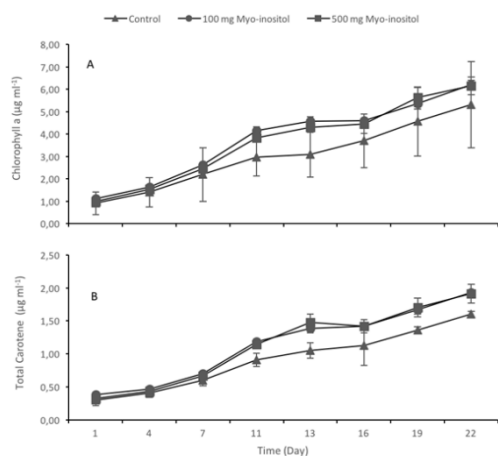
101.00±2.65 × 10<sup>6</sup> cell mL<sup>-1</sup> at 19<sup>th</sup> day and 91.33±2.52 × 10<sup>6</sup> cell mL<sup>-1</sup> at 20<sup>th</sup> day, respectively. Maximum cell number of the control group was reached to 71.12±2.60 × 10<sup>6</sup> cell mL<sup>-1</sup> at 19<sup>th</sup> day. The cell growth showed no difference between 100 mg L<sup>-1</sup> and 500 mg L<sup>-1</sup> of myo-inositol added cultures (P > 0.05). However, the cell density of control group was recorded the lowest, and statistically difference was detected between control and both concentrations of myo-inositol groups (P < 0.05). The maximum specific growth rates were obtained in the first three days for all groups. Mean specific growth rates were calculated for 100 mg L<sup>-1</sup>, 500 mg L<sup>-1</sup> and control group as 0.062, 0.060 and 0.046, respectively (Figure 1B).



**Figure 1.** The cell number and specific growth rate of *N. oculata*; (A) Cell densities of experiments, (B) Specific growth rates of experiments

#### Chlorophyll *a* and total carotene amounts of *N. oculata*

Highest chlorophyll *a* amounts of 100 mg L<sup>-1</sup> and 500 mg L<sup>-1</sup> myo-inositol experimental groups were measured at the day 22 were 6.21±0.20 μg mL<sup>-1</sup> and 6.15±0.39 μg mL<sup>-1</sup> while highest chlorophyll *a* amount of control group was 5.31±0.92 μg mL<sup>-1</sup> (Figure 2A)



**Figure 2.** The chlorophyll *a* and total carotene amounts of *N. oculata*; (A) Chlorophyll *a* amounts of experiments, (B) Total carotene amounts of experiments

Mean chlorophyll *a* amounts per cell were calculated for 100 mg L<sup>-1</sup>, 500 mg L<sup>-1</sup> myo-inositol and the control group as 0.052 pg cell<sup>-1</sup>, 0.052 pg cell<sup>-1</sup> and 0.053 pg cell<sup>-1</sup>, respectively. No significant difference was found between the control group and experimental groups statistically (P > 0.05).

Highest total carotene amounts were measured at the day 22 for all groups. Highest total carotene amount of 100 mg L<sup>-1</sup> experimental group was 1.93±0.04 μg mL<sup>-1</sup> and 500 mg L<sup>-1</sup> myo-inositol experimental group was 1.91±0.14 μg mL<sup>-1</sup>. But total carotene amount of control group was obtained lower than myo-inositol experimental groups as 1.60±0.04 μg mL<sup>-1</sup> (Figure 2B). Mean total carotene amounts per cell were calculated for all groups as 0.016 pg cell<sup>-1</sup>. No significant difference was found between the control group and experimental groups statistically (P > 0.05).

#### DISCUSSION

Many plants, yeast and fungi use inositol as growth agent and protective compounds under stress conditions. Because inositols are rather stable and less vulnerable to degradative enzymes in vivo (Vallurua and Van den Ende, 2011). According to the results of this study, myo-inositol addition to the culture medium of *N. oculata* provide higher cell densities. 100 mg L<sup>-1</sup> and 500 mg L<sup>-1</sup> myo-inositol experimental groups were reached to 1.42-fold cell mL<sup>-1</sup> and 1.28-fold cell mL<sup>-1</sup> than control group, respectively. Statistically, a significant difference was appeared between the control group and experimental groups (P < 0.05), while no statistically significant difference was found between 100 mg L<sup>-1</sup> myo-inositol and 500 mg L<sup>-1</sup> myo-inositol experimental groups (P > 0.05).

In another study, 500 mg L<sup>-1</sup> myo-inositol addition to the culture medium of *Dunaliella salina* resulted in 1.4-fold cell mL<sup>-1</sup> than the control group (Cho et al., 2015). This result shows similarity with *N. oculata* experiments of our study, which differ by effective concentration of myo-inositol. If we compare the content of myo-inositol in the medium that obtained in Cho et al. (2015), we have detected a better result for increasing growth rate of *N. oculata* using as 100 mg L<sup>-1</sup> myo-inositol in the medium. This variability might be attributed to the different species used.

In the present study, both concentration of myo-inositol similarly promoted the cell growth of *N. oculata*, high biomass production was observed with these concentrations and at 100 mg L<sup>-1</sup> maximum cell number was reached to 10.1±0.27 × 10<sup>7</sup> cell mL<sup>-1</sup>. Durmaz et al., (2008) showed that *N. oculata* can use NO<sup>-3</sup> and NH<sup>+4</sup> as sole nitrogen source and was obtained the maximum cell densities of 5.2±0.3 × 10<sup>7</sup> cell mL<sup>-1</sup> and 4.9±0.1 × 10<sup>7</sup> cell mL<sup>-1</sup>, respectively. When the nitrogen concentration was increased 2 times, the cell number was increased 11.2±3.0 × 10<sup>7</sup> cell mL<sup>-1</sup>. So, both myo-inositol addition and increasing the nitrogen concentration in the medium will possibly increase more growth rate of this alga. Bartley et al.,

(2014) showed that *N. oculata* was reached highest cell number as  $95.6 \pm 9.0 \times 10^6$  cell mL<sup>-1</sup> at pH 8 among different pH values varied from 5 to 10. In our study, pH was arranged to 7.5 at the beginning of the experiments. Maximum cell number of control group which was  $71.12 \pm 2.60 \times 10^6$  is lower than Bartley et al., (2014), while 100 mg L<sup>-1</sup> myo-inositol added group was reached higher cell number than mentioned study as  $101.00 \pm 2.65 \times 10^6$  cell mL<sup>-1</sup>.

Mean cell number of *Nannochloropsis* sp. was given as  $75.3 \pm 0.21 \times 10^6$  cell mL<sup>-1</sup> in another study, which is similar to highest cell number of the control group in our study. Also approximately 0.3 pg cell<sup>-1</sup> chlorophyll *a* content determined, which is 6 times higher than mean chlorophyll *a* content of all experiment groups in our study (Vadiveloo et al., 2015). That difference might be occurred because of species and light spectra.

Highest chlorophyll *a* amounts of 100 mg L<sup>-1</sup> myo-inositol, 500 mg L<sup>-1</sup> myo-inositol and control groups were showed the difference and measured as  $6.21 \pm 0.20 \mu\text{g mL}^{-1}$ ,  $6.15 \pm 0.39 \mu\text{g mL}^{-1}$  and  $5.31 \pm 0.92 \mu\text{g mL}^{-1}$ , respectively. However, mean chlorophyll *a* per cell amounts were calculated for experimental groups and control groups as 0.052 pg cell<sup>-1</sup> and 0.053 pg cell<sup>-1</sup>, respectively. These results show that no difference was occurred between all groups by chlorophyll amounts per cell. Also, similar chlorophyll *a* amount per cell of *N. oculata* has been reported in another study (Volkman et al., 1993). Highest total carotene amounts of 100 mg L<sup>-1</sup> myo-inositol, 500 mg L<sup>-1</sup> myo-inositol and control groups were showed the difference and measured as  $1.93 \pm 0.04 \mu\text{g mL}^{-1}$ ,  $1.91 \pm 0.14 \mu\text{g mL}^{-1}$  and  $1.60 \pm 0.04 \mu\text{g mL}^{-1}$ , respectively. However, mean total carotene per cell amounts were calculated for all groups as 0.016 pg cell<sup>-1</sup>. These results show that no difference was occurred between all groups by total carotene amounts per cell. Also, another study on myo-inositol addition into the culture medium of *Dunaliella salina* showed no difference in its pigment composition (Cho et al., 2015).

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Therefore, the production of pigments of *N. oculata* could probably result from nitrogen concentration in the medium. Myo-inositol did not affect the pigment accumulation of *N. oculata*. Besides, pigments level may result from variations in the growth condition (Brown et al., 1999).

## DISCUSSION

*N. oculata* has a big importance in aquaculture hatcheries because of its properties (fatty acid composition, cell size... etc.). This study shows that myo-inositol use in microalgae production could provide higher yields of *Nannochloropsis* cultures. Additionally, at high cell densities, productivity is significantly reduced by the release of and sensitivity to growth inhibitors as reported by Zou and Richmond, (2000).

Nowadays, microalgae are produced intensely and researchers still working on optimizing the culture medium for each species. The main purpose is the increased biomass without any loss of the valuable biomolecules such as fatty acids and pigments. Although the fundamentally biological functions of myo-inositol are still far from clear in plants, in this study, results of chlorophyll *a* and total carotene measurements showed that *N. oculata* keeps those pigments at the same level when cultured in myo-inositol added medium. Yet, we have no information how myo-inositol may affect fatty acid composition of *N. oculata*. Also, optimum myo-inositol concentration should be determined by further studies.

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